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Short communication

## Heterogeneity within the hemagglutinin genes of canine distemper virus (CDV) strains detected in Italy

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### Abstract

Canine distemper virus (CDV) is a highly contagious viral pathogen causing lethal disease in dogs and other mammals. A high degree of genetic variation is found between recent CDV strains and the old CDV isolates used in the vaccines and such genetic variation is regarded as a possible cause of the increasing number of CDV-related diseases in dogs. The H gene shows the greatest extent of genetic variation that allows for distinction of various lineages, according to a geographical pattern of distribution and irrespective of the species of identification. In the present study, hemagglutinin (H) genes obtained from field strains detected from clinical specimens of Italian dogs were analyzed genetically. Phylogenetic analysis revealed that a homogeneous group of CDV strains is widespread in Italian dogs, all which are included into the European lineage. Unexpectedly, strains 179/04 and 48/05 clustered along with CDVs of the Arctic lineage, the highest identity being to strain GR88 (98.0 and 98.4% aa, respectively). The full-length sequence of a red fox CDV strain, 207/00 was also determined and analyzed. The H protein of the fox CDV strain was unrelated to strains within the major European lineage. These results suggest that at least three different CDV lineages are present in Italy.

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**Keywords:** Canine distemper virus; Dogs; Lineages; H gene

### 1. Introduction

Canine distemper virus (CDV) belongs to genus *Morbillivirus* in the Paramyxoviridae family, along with phocine distemper virus (PDV-1) measles virus, rinderpest virus, peste-des-petits-ruminants virus and

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cetacean morbilliviruses. CDV possesses a single-stranded negative RNA that encodes for one envelope-associated protein (M), two glycoproteins (the hemagglutinin/attachment protein H and the fusion

protein F), two transcriptase-associated proteins (the phosphoprotein P and the large protein L), and the nucleocapsid protein N, that encapsidates the viral RNA (van Regenmortel et al., 2000).

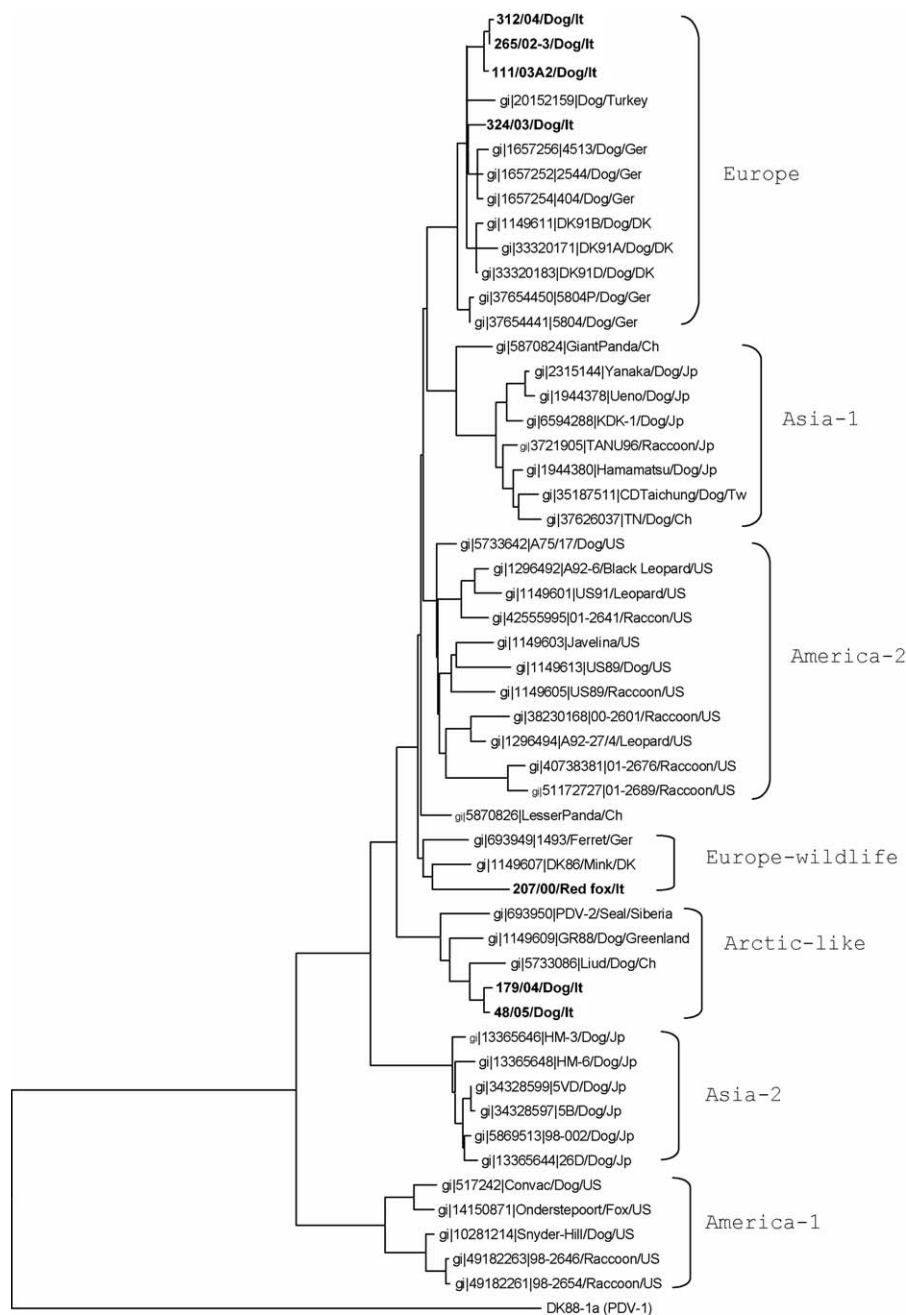


Fig. 1. Phylogenetic relationships between CDV strains on the basis of the nucleotide alignment of the H protein. The phocine strain PDV-1 was used as outgroup. *Abbreviations:* It, Italy; US, United States; Ch, China; Ger, Germany; DK, Denmark; Tw, Taiwan; Tr, Turkey; Jp, Japan.

The disease caused by CDV has been known for centuries. A fascinating historiographic reconstruction by [Blancou \(2004\)](#) hypothesizes that a CDV epidemic spread in the 17th century from the Spanish colonies of Southern America to the European continent.

CDV is a monotypic virus as defined by polyclonal antisera, and a single exposure to the virus confers long-term immunity. The H gene is a key protein for both CDV itself and its animal hosts ([Appel, 1987](#); [Greene et al., 1998](#)) as CDV uses this protein for attachment to receptors on the cell in the first step of infection and an adequate host immune response against the H protein may prevent CDV infection. Comparative studies of CDV strains have revealed that the H gene is subjected to higher genetic/antigenic variation than other CDV genes and that sequence variation may affect neutralization-related sites with disruption of important epitopes ([Blixenkrone-Møller et al., 1992](#); [Haas et al., 1997b](#); [Harder et al., 1993, 1996](#); [Iwatsuki et al., 2000](#); [Örvell et al., 1990](#)). A pronounced genetic diversity in the H gene of recent field CDV isolates has been reported, that may alter the antigenicity of the new strains with respect to the CDV strains that are used currently in the vaccines ([Bolt et al., 1997](#); [Gemma et al., 1996b](#); [Haas et al., 1997a,b](#); [Harder et al., 1993, 1996](#); [Hirama et al., 2004](#); [Iwatsuki et al., 1997, 2000](#); [Mochizuki et al., 1999](#)). The introduction of the live modified CDV vaccines in the 1950s and their extensive use has greatly helped to keep the disease under control ([Appel, 1987](#); [Appel and Summers, 1995](#); [Greene et al., 1998](#)). Notwithstanding, the incidence of CDV-related disease in canine population throughout the world seems to have increased in the last decades and several episodes of CDV disease in vaccinated animals have been reported ([Blixenkrone-Møller et al., 1993](#); [Decaro et al., 2004](#); [Gemma et al., 1996a,b](#); [Kai et al., 1993](#); [Patronek et al., 1995](#); [Scagliarini et al., 2003](#)).

Analysis of CDV strains identified in various geographical settings and from various animal species has revealed that the genetic/antigenic drift acting on the H gene/glycoprotein of CDV is driven mainly by a geographic pattern. Accordingly, a number of major lineages have been identified that accounts for the majority of the CDV strains detected in the field ([Bolt et al., 1997](#); [Carpenter et al., 1998](#); [Haas et al., 1997b](#); [Harder et al., 1996](#); [Iwatsuki et al., 1997](#); [Martella et al., 2002](#); [Mochizuki et al., 1999](#)) (Fig. 1). Herewith,

we describe the genetic characterization of CDVs detected from dogs in Italy. Two unusual CDV strains were detected in the brain tissues of pups with clinical signs of canine distemper. Phylogenetic analysis of the H protein sequence showed that the strains are genetically distinct from the other CDV strains circulating in Italy and Europe. Also, a CDV strain identified in a free-ranging red fox was analyzed and was found to be different from the CDV strains within the major European lineage.

## 2. Materials and methods

### 2.1. *Viruses and clinical specimens*

Nine CDV strains were sequenced in this study. Eight CDV-positive cases were identified by screening animals affected with either neurological signs, enteritis, or respiratory distress. These samples were submitted by animal hospitals in various parts of Southern Italy during the years 2000 and 2004. Samples were initially screened by an indirect immunofluorescence (IF) assay. CDV-specific monoclonal antibodies were used to detect CDV antigens in brain and conjunctive smears, and in Vero cells inoculated with tissue homogenates. Attempts to isolate in tissue cells the viruses were unsuccessful and diagnosis of CDV infection was subsequently obtained by RT-PCR, using primer pair P2-P7 that amplifies a 478-bp-long fragment of the N gene ([Shin et al., 1995](#)). Strain 207/00 was detected in 2000 from free-ranging red foxes with severe neurological signs ([Martella et al., 2002](#)). The profiles of these field CDV strains are summarized in [Table 1](#).

### 2.2. *Reverse transcription and PCR amplification*

Reverse transcription and PCR amplification of the H gene of CDV was achieved as previously described, with minor modifications ([Mochizuki et al., 1999](#)). Total RNA was obtained from 250 mg of tissue homogenates. The RNA was extracted using the RNeasy Kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. The RNA was reverse transcribed with primers CDV-F8 and CDV-R8 and immediately subjected to PCR amplification in a single-step protocol, using SuperScript One-Step RT-

Table 1

List of the CDV strains analyzed in this study

Strain	Species	Age	Origin	Clinical signs	Vaccine
207/00	Red fox	Adult	Lecce	Unknown	No
178/02	Dog	53 days	Bari	Fever + vomit	No
265/02-3	Dog	70 days	Bari	Vomit + diarrhea	No
111/03A	Dog	45 days	Bari	Anorexia + respiratory distress + ocular discharge + enteritis	Yes
111/03B	Dog	45 days	Bari	Anorexia + respiratory distress + ocular discharge + enteritis	Yes <sup>a</sup>
324/03	Dog	6 months	Matera	Nervous signs + ocular discharge	
179/04	Dog	80 days	Messina	Vomit + diarrhea + nervous signs	Yes
312/04	Dog	Adult	Bari	Nervous signs + ocular discharge.	No
48/05	Dog	2 months	Rome	Nervous signs	No

<sup>a</sup> Data not available.

PCR kit (Invitrogen—Life Technologies, Milan, Italy). Reverse transcription was carried out at 48 °C for 60 min, followed by denaturation of the reverse transcriptase at 95 °C for 2 min. Amplification was conducted by a temperature cycling protocol consisting of 35 cycles of 30 s for denaturation at 94 °C, 1 min of primer annealing at 55 °C, and 1 min of extension at 68 °C, followed by 10 min of final extension at 68 °C. To obtain PCR products suitable for cloning, the inner primer pair RH-3 and RH-4 was used to amplify in nested PCR the complete H-gene, using TaKaRa LA Taq polymerase (Cambrex Bio Science Milan, Italy). The temperature cycling protocol consisted of 25 cycles of 1 min of denaturation at 94 °C, 2 min of primer annealing at 50 °C, and 2 min of extension at 68 °C, followed by 2 min of the final extension phase at 68 °C.

### 2.3. Sequencing

The RH3-RH4 PCR products were purified with Ultrafree-DA Columns (Amicon, Millipore). The DNA was sequenced by using the conserved primers RH3 and RH4 and specific primers designed according to an overlapping strategy. The sequences were assembled using Bioedit software package version 2.1 (Hall, 1999) and compared to cognate sequences in the genetic databases using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) and FASTA (<http://www.ebi.ac.uk/fasta33>) web-based programs. The sequences of the H gene of the Arctic-like CDVs are available under accession numbers [DQ226087](#) and [DQ226088](#), for strains 179/04 and 48/05, respectively. The accession number of the H gene

of the fox strain 207/00 is [DQ228166](#). The H gene sequences of the Italian CDV strains 324/03, 265/02-3, and 111/03B are available under accession numbers [DQ494317](#), [DQ494318](#), and [DQ494319](#), respectively.

### 2.4. Phylogenetic analysis

Nucleic acid sequences were aligned with known CDV H gene nucleotide sequences by using Mega 3.0 software package (Kumar et al., 2004). Phocine morbillivirus strain PDV-1 was used as outgroup. The tree was inferred using the Kimura two-parameter model and the neighbor-joining method. Statistical significance of the phylogeny was estimated by bootstrap analysis over 1000 pseudoreplicate data sets. Parsimony analysis was also applied. Heuristic search was carried out by using the Close-Neighbor-Interchange model, by random generation of initial trees. Statistical significance of the phylogeny was estimated by bootstrap analysis over 1000 pseudoreplicate data sets.

## 3. Results

### 3.1. Phylogenetic analysis of amino acid sequence of H genes

Fig. 1 shows the parsimony bootstrap consensus tree displaying the phylogenetic relationships between CDV strains on the basis of the nucleotide alignment of the H gene. The phylogeny inferred with the distance method was consistent with the parsimony analysis. Most Italian CDV strains (178/02, 265/02-3,

111/03A, and 111/03B, 324/03, and 312/04) were clustered within a well-defined lineage (bootstrap value 99%) composed exclusively of European strains. Intra-lineage variation within the major European cluster was <3.5% aa, while variation from the other lineages was >4%.

The fox CDV strain 207/00, along with the mink strain DK86 (Denmark), and the ferret strain 1493, was more distantly related to the major European lineage. The H protein of strain 207/00 had >4.2% aa variation from all CDVs, with exception of strain A75/17 (3.7% aa).

The canine Italian strains 179/04 and 48/05 joined a well-defined clade (bootstrap value 99%), referred to as the Arctic lineage and formed by the phocine strain PDV-2 (Lake Baikal, Siberia) and the canine strains GR88 (Northern Greenland) and Liud (China). Intra-lineage variation was <3.6% aa, while variation from the other lineages was constantly >4% aa.

Two additional well-defined clusters were resolved by phylogenetic analysis, Asia-1 and Asia-2 (bootstrap values of 99 and 83%, respectively). Lineage Asia-1 consists of Japanese and Chinese strains, KDK-1-like. Lineage Asia-2 includes only Japanese strains (prototype strain 98-002). The strains within each of such lineages were tightly related to each other (<2% aa variation), while variation from other lineages was constantly >4% in both cases.

Old CDV strains, Onderstepoort, Convac, and Snyder Hill, all which were isolated in 1930–1950s, and the raccoon strains 98-2654 and 98-2646, detected in 1998, form a distinct clade (bootstrap value 99%). Amino acid sequence variation within this lineage is <4.2%, while aa variation from CDV strains of other lineages is >8%. As all the strains were isolated in the American continent, the lineage is designated as America-1. A number of American strains detected from various animal species (raccoon, dog, and wild felids) were clustered in a second lineage (bootstrap value 95%), termed America-2.

### 3.2. Amino acid sequence analysis of H genes of the Italian CDV strains

The H gene of the Italian CDV strains was 1824 nt long and the inferred amino acid sequence was 607 aa long. The H genes of strains 111/03A and 111/03B were 100% identical to each other at the nt

level. Similar, the H gene of strain 265/02-3 was 100% nt identical to strain 312/04 and 178/02. Therefore, only strain 111/03B and 265/02-3 were included for sequence comparison. Strain 324/03, 265/02-3, and 111/03B displayed the highest sequence identity to the European strains (98.3–99.0% nt and 97.0–99.0% aa), while identity to each others ranged from 99.8–98.1% aa and 99.7–96.3% nt. Identity to lineage America-1 (Onderstepoort-like) was <92.5 nt and 91.5% aa. A total of eight potential glycosylation sites were recognized at positions 19–21, 149–151, 309–311, 391–393, 422–424, 456–458, 587–589, and 603–605. All such glycosylation sites are conserved in CDVs within the major European lineage, while site 309–311 is missing in CDV strains of lineage America-1 (that includes the vaccines) and the glycosylation site 603–605 is missing in strain Onderstepoort due to a 9-nucleotides deletion at the 3' end of the H gene (Fig. 2).

The fox strain 207/00 displayed the highest identity (96.3% aa and 96.3% nt) to strain A75/17, isolated in USA in 1975. Identity to the European CDVs ranged from 94.6 to 95.8% aa and 95.1 to 96.2 nt. Identities to the atypical European strains DK86 (mink) and 1493 (ferret) were 95.8% aa (96.3% nt) and 95.0% aa (95.8% nt), respectively. A total of seven potential glycosylation sites were present in strain 207/00, because of the aa substitution 589-Thr to Ala, that disrupts the NXT site 587–589 (Fig. 2).

Strain 179/04 and 48/05 were 99.4 aa and 99.6 nt identical to each other. Strain 179/04 showed the highest identity (96.4–98.0% aa and 97.0–98.1% nt) to the Arctic strains GR88, Liud, and PDV-2. Strain 48/05 displayed the highest identity to strain GR88 (98.4% aa and 97.7% nt). Identity to the European strains ranged from 93.0 to 94.7% aa (94.2 to 95.2% nt), while identity to strains of the lineage America-1 ranged from 90.8 to 91.7% aa (92.3 to 92.7% nt). Eight potential glycosylation sites, conserved in all the Arctic strains, were recognized at positions 19–21, 149–151, 309–311, 391–393, 422–424, 456–458, 587–589, and 603–605. A number of synapomorphies (shared-derived aa residues) were found in the Arctic CDV strains, i.e. 20-L, 82-D, 165-L, 198-S, 222-S, 266-F, 370-N, and 371-L (Fig. 2).



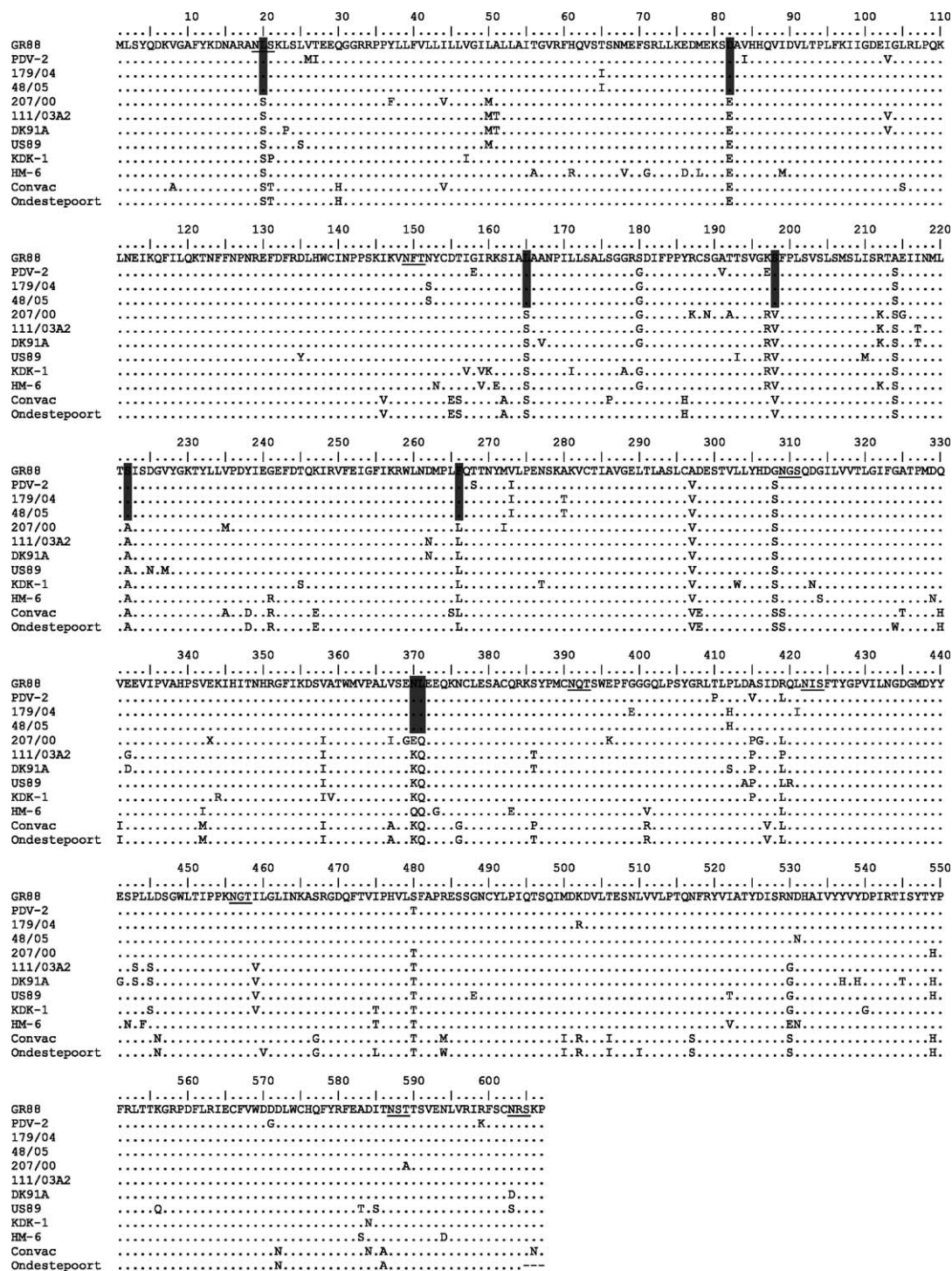


Fig. 2. Amino acid sequence alignment of the H proteins of various CDV strains, representative of the major lineages. The potential glycosylation in the H protein of the Arctic strains are underscored. Synapomorphies among CDV strains of the Arctic lineage are shadowed.

#### 4. Discussion

Most CDV strains detected from dogs during the survey in Italy displayed a high genetic homogeneity within the European lineage, the highest aa sequence identity (99.0%) being to the canine strain 5804, detected in Germany in 1990. Unexpectedly, however, the H gene of two CDV strains, 179/04 and 48/05, were more tightly related (up to 98.4% aa) to CDVs of the Arctic lineage, that includes the canine strain GR88 detected in Northern Greenland in 1988 (Blixenkrone-Møller et al., 1992), the phocine strain PDV-2, identified in 1988 from a Siberian seal (*Phoca siberica*) of Lake Baikal (Visser et al., 1990) and the canine strain, isolate Liud, detected in China in the mid 1990s. The initial description of Arctic viruses dates back to the late 1980s, when morbillivirus-related epizootics were observed in seals in Northern Europe and Siberia (Likhoshway et al., 1989; Osterhaus and Vedder, 1988; Titenko et al., 1990; Visser et al., 1990). Subsequent genetic analysis demonstrated that the 1987 and 1988 epizootics were epidemiologically distinct, as they were caused by a phocine distemper virus, PDV-1 and by a CDV-like strain, PDV-2, respectively. Also, PDV-2-like strains continued to circulate in Baikal seals at least until 1992 and feral and domestic dogs around lake Baikal were suspected to be the source of infection for the freshwater seals, even if the presence of PDV-2-like CDVs was not assessed directly in dogs (Mamaev et al., 1995). Noteworthy, almost in the same years, 1988, a CDV strain, GR88, was detected from a virgin soil outbreak in a sledge dog population in remote Inuit settlement of arctic Northern Greenland (Blixenkrone-Møller et al., 1992), that was subsequently found to be closely related to strain PDV-2. Accordingly, both the strains were regarded as a separate lineage circulating across the Arctic ecosystem in susceptible species, such as polar bear and arctic foxes (Bolt et al., 1997; Cattet et al., 2004; Haas et al., 1997b; Harder and Osterhaus, 1997). The Italian Arctic strain 179/04 was detected from a 3-months pup in Sicily, in Southern Italy. The pup had been vaccinated (at 8 weeks of age) and the disease was characterized by a gastroenteric form and by nervous symptoms. Strain 48/05 was detected from a non-vaccinated 2-months pup in Rome, affected by nervous signs. The evidence that the pups were

infected and killed by Arctic-like CDV strains in separate geographic settings raises interrogatives on the origin and diffusion of these unusual CDV strains. A possible explanation is that an Arctic-like strain was introduced by other dogs imported into Italy from Eastern Europe or Northern Asia and that the strain steadily spread across canine population. This fact highlights the constant threat to dogs represented by uncontrolled trading of low cost and high value breed pets, a phenomenon that has been intensifying in the last decades in Italy. Larger epidemiological surveys are required to understand whether the unusual Arctic-like CDV strains identified in Italy have got permanently established in canine population in Italy or they represent occasional findings.

The full-length H gene sequence of a CDV strain detected in a red fox was also determined. Strain 207/00 was detected in 2000 from free-ranging red foxes with severe neurological signs (Martella et al., 2002). The fox CDV strain was distantly related (95.8–96.6% aa) to the CDV strain of the European lineage, including the CDV strains detected in the Italian dogs, suggesting the existence of non-urban epidemiological cycles that maintain atypical CDVs in the wildlife.

Due to the few epidemiological surveys and to the different genes targeted in the various studies, the distribution of the major CDV lineages throughout the world is not clear. For instance, the fact that the old CDV strains, still used in the vaccines, and distantly related from the novel CDVs, have apparently disappeared in the last five decades, has led to the proposition that such strains no longer exist in the field. The Snyder Hill strain was isolated in Ithaca, NY, USA in the 1950s from the brain of a dog and passaged in vivo in dogs before being adapted to cell growth in NL-DKC cells (Brown et al., 1972). The Onderstepoort strain, used worldwide as an attenuated live vaccine, dates back to a disease outbreak among North American ranches foxes in the 1930s (Haig, 1956). Monitoring of raccoon population in Chicago area has revealed that an epidemic occurred in 1998 was determined by CDV strains tightly related to the old CDV strains (Onderstepoort-like) of the lineage America-1 (Lednicky et al., 2004). Harder and Osterhaus (1997) have reported the identification of an Onderstepoort-like CDV strain from a dog in Northern Ireland. In addition, by analysis of the NP gene, the identification of CDV strains related to the Onderstepoort strain has been reported in Thailand and



Poland (Keawcharoen et al., 2005; Rzeutka and Mizak, 2003). Such findings may be accounted for by a reversion of pathogenicity in vivo of vaccine strains, or, alternatively, by the persistence in the field of the CDV lineage America-1.

Whether the effectiveness of the currently employed vaccines may be partially compromised by the extent of genetic/antigenic variation observed is unclear. It has been shown that sera raised against wild-type CDV isolates have neutralizing titers up to 10-fold greater against the homologous virus than against vaccine strains of CDV (Harder et al., 1996). Accordingly, the genetic/antigenic drift observed in currently circulating CDVs should be considered as a possible factor leading to the resurgence of distemper cases in vaccinated dog populations. Analysis of CDV strains detected globally and from a variety of host species will provide a more in-depth understanding of the global ecology of CDV and will provide the basis for improvement of current CDV vaccines.

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